conditioning or test trials. Furthermore, the authors had participants report if they saw a face on each trial; in fact participants had to make a two-alternative forced-choice decision whether the face was male or female — participants' decisions were just below chance. After this discrimination task participants had to rate the confidence of their choice — confidence ratings were no higher on correct trials than incorrect trials.

The conditioning or learning effects outside of awareness reported by Raio *et al.* [3] display some distinct characteristics that differentiate them from learning with awareness. The learning effects appeared very rapidly and subsequently diminished very rapidly. Unlike normal learning these effects faded during further conditioning, whereas typically in this kind of conditioning experiment the learning would continue before stabilising. Such brevity in associative learning dynamics is clearly distinct from typical conditioning effects, which often last for days. Might this learning outside of awareness be tapping into a categorically different learning mechanism, or perhaps a subset of normal learning processes? This is an interesting idea that is compatible with the data in the new study [3].

Raio *et al.* [3] did include a fully visible condition, which showed a very different temporal learning profile. In their visible condition, however, both the learning and test-trials were both visible, while in the unaware condition both the training and test-trials were invisible. Hence, we do not have a conscious measure of conditioning outside of awareness, only an unconscious one. To help clarify the underlying mechanism what is needed is a third condition in which only the test trials are visible while the training trials remain suppressed from awareness. Such an experiment would help tease apart the nature of this unconscious learning.

Previous claims of unconscious conditioning have been criticised on a number of methodological grounds such as trial sequence artifacts, failure to assess participant hypotheses, and insensitivity to partial awareness [2]. In fact, some researchers have gone so far as to argue that all conditioning involves cognitive representation and hence conscious awareness [12]. Others maintain that conditioning is carried out by a separately evolved specialised system [13,14]. Will continuous flash suppression finally provide the experimental tool to resolve this long-standing debate? Watch this space!

Associative learning is thought to form the backbone of the mechanisms of many psychological disorders and their treatments [15,16]. Many behavioural interventions for psychological disorders rely on counterconditioning or extinction-like approaches, such as cognitive behavioural therapy. Does this new paper by Raio *et al.* [3] shed light on any new clinical treatment possibilities? Potentially yes, if mechanisms of associative learning can operate without awareness, it is possible to imagine a future non-intrusive treatment option that might be run on patients without their conscious involvement. However, the brief lifetime of the effects in the new paper might limit any potential clinical applications.

References

1. Carew, T.J., Walters, E.T., and Kandel, E.R. (1981). Classical conditioning in a simple withdrawal reflex in Aplysia californica. J. Neurosci. *1*, 1426–1437.
2. Lovibond, P.F., and Shanks, D.R. (2002). The role of awareness in Pavlovian conditioning: empirical evidence and theoretical implications. J. Exp. Psychol. Anim. Behav. Proc. *28*, 3–26.
3. Raio, C., Carmel, D., Carrasco, M., and Phelps, E.A. (2012). Nonconscious fear is quickly acquired but swiftly forgotten. Curr. Biol. *22*, R477–R479.
4. Tsuchiya, N., and Koch, C. (2005). Continuous flash suppression reduces negative afterimages. Nat. Neurosci. *8*, 1096–1101.
5. Tsuchiya, N., Koch, C., Gilroy, L.A., and Blake, R. (2006). Depth of interocular suppression associated with continuous flash suppression, flash suppression, and binocular rivalry. J. Vis.n *6*, 1068–1078.
6. Stein, T., Hebart, M.N., and Sterzer, P. (2011). Breaking continuous flash suppression: a new measure of Uunconscious processing during interocular suppression? Front. Hum. Neurosci. *5*, 167.
7. Pearson, J., and Clifford, C.W. (2005). Suppressed patterns alter vision during binocular rivalry. Curr. Biol. *15*, 2142–2148.
8. Blake, R., Tadin, D., Sobel, K.V., Raissian, T.A., and Chong, S.C. (2006). Strength of early visual adaptation depends on visual awareness. Proc. Natl. Acad. Sci. USA *103*, 4783–4788.
9. Pasley, B.N., Mayes, L.C., and Schultz, R.T. (2004). Subcortical discrimination of unperceived objects during binocular rivalry. Neuron *42*, 163–172.
10. Almeida, J., Mahon, B.Z., Nakayama, K., and Caramazza, A. (2008). Unconscious processing dissociates along categorical lines. Proc. Natl. Acad. Sci. USA *105*, 15214–15218.
11. Jiang, Y., Costello, P., Fang, F., Huang, M., and He, S. (2006). A gender- and sexual orientation-dependent spatial attentional effect of invisible images. Proc. Natl. Acad. Sci. USA *103*, 17048–17052.
12. Mitchell, C.J., De Houwer, J., and Lovibond, P.F. (2009). The propositional nature of human associative learning. Behav. Brain Sci. *32*, 183–198, discussion 198–246.
13. Clark, R.E., and Squire, L.R. (1998). Classical conditioning and brain systems: the role of awareness. Science *280*, 77–81.
14. Ohman, A., and Mineka, S. (2001). Fears, phobias, and preparedness: toward an evolved module of fear and fear learning. Psychol. Rev. *108*, 483–522.
15. Rauch, S.L., Shin, L.M., and Phelps, E.A. (2006). Neurocircuitry models of posttraumatic stress disorder and extinction: human neuroimaging research–past, present, and future. Biol. Psych. *60*, 376–382.
16. Milad, M.R., Rauch, S.L., Pitman, R.K., and Quirk, G.J. (2006). Fear extinction in rats: implications for human brain imaging and anxiety disorders. Biol. Psychol. *73*, 61–71.

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DOI: 10.1016/j.cub.2012.04.042

Nuclear Positioning: Dynein Needed for Microtubule Shrinkage-Coupled Movement

Nuclear movement often requires interactions between the cell cortex and microtubules. A new study has revealed a novel protein interaction linking microtubule plus-ends with the cortex and a role for dynein in microtubule shrinkage-coupled movement.

Xin Xiang

Proper positioning of nuclei and mitotic spindles is crucial for the normal growth and development of many eukaryotic organisms [1]. Unlike other cellular organelles that move along microtubule tracks, nuclei/spindles move in response to either pushing or pulling force on the microtubules

whose minus-ends are physically linked with the nuclei/spindles. Pushing or pulling force is often generated through interactions of growing (polymerizing) or shrinking (depolymerizing) microtubule plus-ends with the cell cortex, but the mechanistic details of these interactions are not clear. A microtubule's 'end-on' interaction with the cortex can be non-productive if it is simply followed by catastrophe (the switch from growth to shrinkage) and detachment from the cortex. Cortex-trigged catastrophe is a commonly observed phenomenon likely caused by the 'barrier effect' based on diminished on-rate of GTP–tubulin subunits [2]. However, incorporation of GTP–tubulin subunits to the plus ends at the cortex can generate pushing force for nuclear movement [3–5]. It is not clear whether additional linker proteins are required for the pushing mechanism *in vivo*. For generation of microtubule shrinkage coupled pulling force, a link between the shrinking plus-end and the cortex is strictly required but its identity remains to be elucidated. Importantly, a new study by ten Hoopen *et al.* [6], reported in this issue of *Current Biology*, has identified a novel physical interaction between Bim1 (budding yeast homolog of EB1, a microtubule plus-end-tracking protein) at the microtubule plus-end and Bud6 at the cortex, which is required for growth/shrinkage-coupled nuclear motility. Furthermore, this study has revealed a new *in vivo* role of cytoplasmic dynein, a minus-end-directed microtubule motor, in microtubule shrinkage-coupled nuclear movement.

Nuclear/spindle positioning in budding yeast utilizes several mechanisms operating at different cell cycle stages [4,5,7]. During G1/S, the old spindle pole body (SPB) emanates cytoplasmic microtubules that probe the cortex of the small bud. Bud6, an actin-interacting protein localized at the bud cortex captures microtubules to cause movements of the SPB and its attached nucleus [6]. Previously, a microtubule plus-end depolymerase, Kip3 (kinesin-8), was found to play an important role in nuclear positioning [8]. Loss of Kip3 causes excessive microtubule growth at the cortex, which causes the nucleus to be pushed back to the mother cell [8]. Interestingly, the new study found that Bud6 is required for the excessive microtubule growth in the absence of Kip3, indicating that the plus-ends captured by Bud6 are permitted to grow at the cortex to generate pushing force. The microtubule plus-end-tracking protein Bim1 is also required for the Bud6-dependent capture mechanism. Rather than being indirectly involved by enhancing a microtubule's searching ability, Bim1 directly interacts with Bud6, and this interaction is crucial for the plus-end–cortex link required for SPB movements. Importantly, microtubule shrinkage-coupled SPB movements depend on dynein [6]. Dynein is known to pull the anaphase spindle into the bud neck [4,5] using a 'microtubule-sliding' mechanism rather than a 'capture/shrinkage' mechanism [4]. During anaphase, the dynein tail binds to its cortical anchor, Num1, and the motor heads walk along an engaged astral microtubule towards its minus-end, causing the microtubule to slide along the bud cortex [4,7]. Interestingly, Num1 is not required for SPB movement at G1/S, which uses the capture/shrinkage mechanism [6]. During SPB movement toward the cortex, dynein is accumulated at the shrinking end in contact with the cortex, and this accumulation appears to be crucial for shrinkage-coupled SPB movement [6]. Thus, dynein seems to play a Num1-independent role in tethering the shrinking plus-end to pull the minus-end-attached SPB toward the cortex.

Remarkably, a direct role of dynein in tethering the dynamic microtubule plus-ends has been demonstrated by two recent studies in minimal *in vitro* systems using either purified yeast dynein attached to microfabricated barriers [9] or purified bovine brain dynein bound to optical trap-controlled beads [10]. The requirement of ATP indicates that dynein's ability to walk is important for its tethering function [9,10]. Most relevant to the new study [6] is that barrier-anchored yeast dynein is sufficient for generating significant pulling force on the captured microtubule [9]. Dynein is unlikely to function as a plus-end depolymerase [9]. However, the ability of the anchored dynein to pull by walking may cause the plus-end to hit the barrier, and the particular geometry of the interaction may facilitate catastrophe due to the barrier effect [2,9]. *In vivo*, more players are involved. The Bud6–Bim1 interaction is required for the initial capture of growing microtubules [6], and it remains to be dissected whether it is continuously required after shrinkage is initiated. The plus-end depolymerase Kip3 contributes to the capture/shrinkage mechanism by enhancing catastrophe [6,8]. A plus-end depolymerase can in theory be a major player in the capture/shrinkage mechanism if its connections with the cortex and the plus-end are maintained during shrinkage. Indeed, Kar3 (kinesin-14), a minus-end-directed kinesin with a plus-end depolymerase activity [11], is critical for dynein-independent nuclear movement towards the yeast mating protrusion, and its accumulation persists at the plus-end–cortex junction during shrinkage [12]. However, the accumulation of Kip3 disappeared from the shrinking end contacting the cortex in the most recent study [6], and thus Kip3 is unlikely to continue its depolymerizing function, especially given the cooperative mechanism of Kip3 action [13]. In contrast, dynein accumulation persists at the shrinking end contacting the cortex [6], and dynein seems to be a key player in the capture/shrinkage mechanism [6]. However, the identity of dynein's cortical anchor and the mechanism of anchoring are unclear.

As mentioned earlier, a link between the shrinking end and the cortex is essential for pulling-force generation. If a plus-end-tracking protein is required as a structural component of the link, it must interact not only with the growing end but also with the shrinking end. Although EB1 and its homologs in other systems only track the growing ends, Bim1 tracks both the growing and shrinking ends [6,14]. The mechanism behind this phenomenon remains a total mystery. Dynein and CLIP-170 (a microtubule plus-end tracking protein) homologs in the filamentous fungus *Aspergillus nidulans* and budding yeast are also able to track both growing and shrinking ends [15–19], and the accumulation at the shrinking ends is correlated with the requirement of plus-end-directed kinesins [17–19]. In

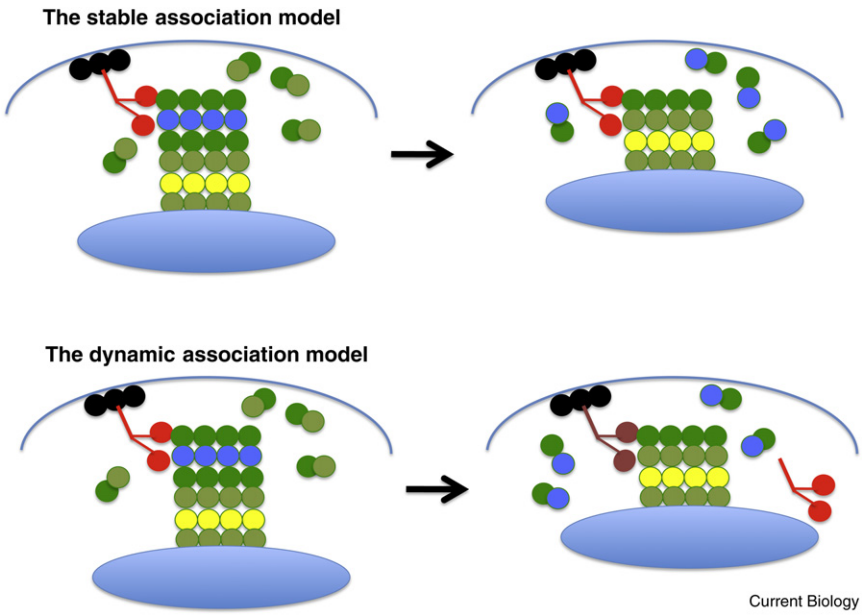

Figure 1. Two models of dynein–cortex interactions during microtubule shrinkage-coupled nuclear movement.

The microtubule plus-end faces the cortex and the minus-end is linked to the nucleus. Cortically anchored dynein, with its minus-end-directed motor activity that pulls the microtubule toward the cortex, enhances catastrophe due to the barrier effect [9]. Microtubule shrinking velocity is relatively low, and the shrinking end is not detached from dynein during shrinkage [9]. In the stable association model, a cortical anchor protein (black circle) associates with the same dynein molecule at two different time points during shrinkage. In the dynamic association model, the cortical interaction with a dynein molecule (red) at the first time point is lost but quickly replaced by a new interaction with a different dynein molecule (brown) at the second time point. For simplicity, only one dynein molecule is depicted. In the cell, however, dynein molecules accumulate at the shrinking end, allowing multiple consecutive interactions to maintain the link between the shrinking end and the cortex.

the budding yeast and the dimorphic fungus *Ustilago maydis,* analyses of fluorescence recovery after photobleaching of Bik1 (yeast CLIP-170 homolog) and dynein revealed that these proteins are highly dynamic at the plus ends, even when the ends are stable [18,20]. It is likely that dynein's shrinking-end accumulation described in the new paper [6] is also dynamic, with new molecules constantly arriving to replenish the population, as the previous members are lost with the old end. Thus, two distinct models can be envisioned for dynein–cortex interaction during plus-end shrinkage-coupled nuclear movement (Figure 1). The 'stable association' model involves a stable association between a dynein molecule and its cortical anchor, similar to the situation in the *in vitro* system [9]. For this model, while the growing-end accumulation of dynein may facilitate delivery of dynein to its cortical anchor [7,16], the shrinking-end accumulation would seem unnecessary. In the 'dynamic association' model, old interactions are constantly being broken and replaced by new interactions between the cortex and newly arrived dynein molecules at the new end. The accumulation of dynein at the shrinking end would allow multiple interactions that are unlikely to be broken all together at any particular time point, and such a collective effort of the plus-end molecules benefits cortical tethering of the shrinking end. In addition, the Bud6–Bim1 interaction could possibly serve as an additional tether, which may also involve dynamic interactions of multiple Bim1 molecules at the shrinking end with multiple Bud6 molecules at the Bud6-marked cortical site. Finally, one interesting possibility not excluded is that Bim1 indirectly interacts with the dynein tail via several other proteins at the shrinking end, thereby linking dynein to Bud6 at the cortex.

References

1. Morris, N.R. (2000). Nuclear migration. From fungi to the mammalian brain. J. Cell Biol. *148*, 1097–1101.
2. Janson, M.E., de Dood, M.E., and Dogterom, M. (2003). Dynamic instability of microtubules is regulated by force. J. Cell Biol. *161*, 1029–1034.
3. Tran, P.T., Marsh, L., Doye, V., Inoue, S., and Chang, F. (2001). A mechanism for nuclear positioning in fission yeast based on microtubule pushing. J. Cell Biol. *153*, 397–411.
4. Adames, N.R., and Cooper, J.A. (2000). Microtubule interactions with the cell cortex causing nuclear movements in Saccharomyces cerevisiae. J. Cell Biol. *149*, 863–874.
5. Yeh, E., Yang, C., Chin, E., Maddox, P., Salmon, E.D., Lew, D.J., and Bloom, K. (2000). Dynamic positioning of mitotic spindles in yeast: role of microtubule motors and cortical determinants. Mol. Biol. Cell *11*, 3949–3961.
6. ten Hoopen, R., Cepeda-García, C., Fernandez-Arruti, R., Juanes, M.A., Delgehyr, N., and Segal, M. (2012). Mechanism for astral microtubule capture by cortical Bud6p priming spindle polarity in S. cerevisiae. Curr. Biol. *22*, 1075–1083.
7. Markus, S.M., and Lee, W.L. (2011). Microtubule-dependent path to the cell cortex for cytoplasmic dynein in mitotic spindle orientation. BioArchitecture *1*, 1–7.
8. Gupta, M.L., Jr., Carvalho, P., Roof, D.M., and Pellman, D. (2006). Plus end-specific depolymerase activity of Kip3, a kinesin-8 protein, explains its role in positioning the yeast mitotic spindle. Nat. Cell Biol. *8*, 913–923.
9. Laan, L., Pavin, N., Husson, J., Romet-Lemonne, G., van Duijn, M., Lopez, M.P., Vale, R.D., Julicher, F., Reck-Peterson, S.L., and Dogterom, M. (2012). Cortical dynein controls microtubule dynamics to generate pulling forces that position microtubule asters. Cell *148*, 502–514.
10. Hendricks, A.G., Lazarus, J.E., Perlson, E., Gardner, M.K., Odde, D.J., Goldman, Y.E., and Holzbaur, E.L. (2012). Dynein tethers and stabilizes dynamic microtubule plus ends. Curr. Biol. *22*, 632–637.
11. Sproul, L.R., Anderson, D.J., Mackey, A.T., Saunders, W.S., and Gilbert, S.P. (2005). Cik1 targets the minus-end kinesin depolymerase kar3 to microtubule plus ends. Curr. Biol. *15*, 1420–1427.
12. Maddox, P.S., Stemple, J.K., Satterwhite, L., Salmon, E.D., and Bloom, K. (2003). The minus end-directed motor Kar3 is required for coupling dynamic microtubule plus ends to the cortical shmoo tip in budding yeast. Curr. Biol. *13*, 1423–1428.
13. Varga, V., Leduc, C., Bormuth, V., Diez, S., and Howard, J. (2009). Kinesin-8 motors act cooperatively to mediate length-dependent microtubule depolymerization. Cell *138*, 1174–1183.
14. Wolyniak, M.J., Blake-Hodek, K., Kosco, K., Hwang, E., You, L., and Huffaker, T.C. (2006). The regulation of microtubule dynamics in Saccharomyces cerevisiae by three interacting plus-end tracking proteins. Mol. Biol. Cell *17*, 2789–2798.
15. Han, G., Liu, B., Zhang, J., Zuo, W., Morris, N.R., and Xiang, X. (2001). The Aspergillus cytoplasmic dynein heavy chain and NUDF localize to microtubule ends and affect microtubule dynamics. Curr. Biol. *11*, 719–724.
16. Sheeman, B., Carvalho, P., Sagot, I., Geiser, J., Kho, D., Hoyt, M.A., and Pellman, D. (2003). Determinants of S. cerevisiae dynein localization and activation: implications for the mechanism of spindle positioning. Curr. Biol. *13*, 364–372.
17. Zhang, J., Li, S., Fischer, R., and Xiang, X. (2003). Accumulation of cytoplasmic dynein and dynactin at microtubule plus ends in Aspergillus nidulans is kinesin dependent. Mol. Biol. Cell *14*, 1479–1488.

18. Carvalho, P., Gupta, M.L., Jr., Hoyt, M.A., and Pellman, D. (2004). Cell cycle control of kinesin-mediated transport of Bik1 (CLIP-170) regulates microtubule stability and dynein activation. Dev. Cell *6*, 815–829.
19. Efimov, V.P., Zhang, J., and Xiang, X. (2006). CLIP-170 homologue and NUDE play overlapping roles in NUDF localization in Aspergillus nidulans. Mol. Biol. Cell *17*, 2021–2034.
20. Schuster, M., Kilaru, S., Ashwin, P., Lin, C., Severs, N.J., and Steinberg, G. (2011). Controlled and stochastic retention concentrates dynein at microtubule ends to keep endosomes on track. EMBO J. *30*, 652–664.

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DOI: 10.1016/j.cub.2012.04.044

Single-Molecule Imaging: A Collagenase Pauses before Embarking on a Killing Spree

Single-molecule tracking provides new insights into how an ATP-independent endo-proteolytic machine digests collagen fibrils during their remodeling.

Gwangrog Lee[1] and Taekjip Ha[2,*]

The extracellular matrix is a well-organized macromolecular platform that specifies the mechanical properties of connective tissues to maintain the cell shapes. Collagen is a major element of the extracellular matrix and is the most abundant protein in human tissues. Somewhat like the art of knitting, collagen is weaved into protein strings to form collagen fibrils, which then form a lattice (Figure 1A), which are highly resistant to proteolytic degradation. Over time, however, this highly stable scaffold must undergo remodeling during pathophysiological processes, such as wound healing, tumor progression, metastatic invasion, and host defense mechanisms [1]. Matrix metalloproteases (MMPs) are the endopeptidases in charge of degrading collagen fibrils, hence called 'collagenases', and their activities must be tightly regulated. Although it is now known that other types of processive proteases, e.g. ClpXP, use chemical energy derived from ATP hydrolysis to mechanically unfold protein structures before digestion [2,3], it has been a puzzle how MMPs can help remodel stable organizations of collagen without using additional energy sources.

Studying native collagen fibrils is difficult using traditional enzymology tools because the extended substrate is insoluble and heterogeneous. In 2004, a new approach of fluorescence correlation spectroscopy that examines molecular diffusion on a sub-micron scale was applied to the study of an MMP subtype, MMP1, and led to the proposal of a Brownian ratchet model; MMP1 diffuses on type 1 collagen but its Brownian motion is biased through a 'burnt bridge' effect caused by collagen proteolysis [4]. But, because of the difficulty in handing native collagen samples and the technical limitations of averaging over many molecules, the earlier study could not address how MMPs initiate and carry out the degradation of the native substrate. Now, in this issue of *Current Biology*, Sarkar *et al.* [5] report the use of single-molecule fluorescence imaging to shed new light on these issues and provide a major leap in our understanding of the multiple phases of native collagen degradation. Fluorescently labeled MMP1 proteins were added to native collagen fibrils immobilized on the sample cell surface and the motion of single MMP1 molecules on the fibrils was monitored in real time through total internal reflection fluorescence microscopy.

As anticipated, the authors found that MMP1 diffuses on the collagen fibrils. But direct imaging allowed them to show that the motion is one-dimensional (1D), occurring along the collagen fibril, but not across fibrils, raising the possibility that MMP1 uses 1D diffusional search to find the cleavage sites on the 3D collagen lattice. Interestingly, MMP1's 1D diffusion was not continuous but was punctuated by pauses. In fact, MMP1 spent ~90% of the time in paused states with only ~10% of time spent transiting between different pausing sites. As a result, these pauses dominate the overall diffusion timescales. One class of pauses followed a single exponential distribution of their lifetimes and occupied no special positions on the fibrils. The second class of pauses was longer in duration and had a distinct lag phase before escaping the paused state. Furthermore, statistical analysis showed that multiple sequential steps are necessary before the escape (Figure 1B). The molecular origin of these class II pauses that exhibit the lag phase is as yet unknown but these pauses are reminiscent of the activity of nucleic acid enzymes that can accumulate elastic energy through in multiple irreversible reactions before transitioning to a subsequent phase [6–8]. Furthermore, these class II pauses occur at periodic locations (see below).

As the enzyme escapes the class II pause site, it shows a so-called 'ballistic' behavior with a distinct bias in its initial motion along one fibril direction. This biased random walk was not observed with an active site mutant of MMP1, suggesting that the directional bias is related to the endopeptidase activity of MMP1. Furthermore, the ballistic behavior was observed at 37°C but not at 25°C. These observations led to an intriguing possibility that thermally induced local unfolding of collagen may allow MMP1 to initiate the collagenolysis. Upon initiation, cleavage reaction would bias the diffusion by burning the bridge behind so that subsequent diffusion appears ballistic along the collagen fibril. To obtain quantitative details of the collagenolysis the authors performed modeling and simulations. They found that only 5% of class II pauses result in the actual initiation of cleavage but this killing rampage is highly processive and, on average, 15 consecutive cleavage events result from one initiation event. MMP1 spends ~90% of its time at pausing states due to the inaccessibility of the cleavage sites, but once the first cleavage occurs, subsequent cleavages progress rapidly as the